# Formulation, characterization and release studies of alginate microspheres encapsulated with tetanus toxoid for nasal immunization

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## Abstract

Alginate is a safe, non-immunogenic and inexpensive natural polymer with high mucoadhesive properties. Alginate microspheres can be used as a delivery system for antigens to the mucosal surfaces. Alginate microspheres were prepared by emulsification-internal phase gelation. Effects of different sonication times; alginate, emulsifier and calcium concentrations; and the volume of calcium solution on mean size, size range, surface roughness and porosity, sphericity and clumping of microspheres were evaluated using optical microscope and zetasizer. The best conditions were resulted by 90 sec. sonication, 3% alginate solution, 2% surfactant, and 60 ml of 0.33% $CaCl_2$ in octanol. The resulting microspheres had mean size of $1.34 \pm 0.3$ μm (n=3) and size range of $0.3 – 2.0$ μm with no surface roughness and porosity, low clumping and high sphericity. The encapsulation rate was about 47.7% (n=6). All batches showed nearly the same release profiles with a low burst release. The intacticity of model antigen (tetanus toxoid=TT) extracted from microspheres was confirmed by SDS-PAGE and the antigenicity of TT was studied by ELISA and found to be $91 \pm 5\%$ that of the original TT. With regard to the size and morphological characteristics of the prepared microspheres and preservation of the antigenicity of the encapsulated TT, they could be used as a delivery system for mucosal delivery of TT.

Keywords: alginate, microsphere, mucosal delivery, tetanus toxoid.

## Introduction

Alginic acid is a linear anionic polysaccharide composed of β-D-mannuronic acid and D-gluronic acid. This hydrophillic polysaccharide has a high molecular weight (80-290 KDa) and primarily extracted from three species of brown algae. Sodium alginate  is slowly soluble in water and its solutions are most stable between pH 4 and 10. Alginic acid precipitates below pH 3 and the pH of its 1% w/v solution is 7.2. (5,12,13).

Sodium alginate could be cross-linked by divalent ($Ca^{2+}$, $Ba^{2+}$, $Zn^{2+}$, ….) or trivalent ($Al^{3+}$, …) cations. These complexes are respectively two and three dimensional (1,10). The reactivity with calcium and resulting water insoluble Ca-alginate gel is a direct function of the average chain length of guluronic acid. So alginates with higher percentage of guluronic acid show more ability to form gel (19).

Microspheres prepared from Ca-alginate have a good potential to be used as carrier for antigen delivery to mucosal membranes. Of great benefits of alginate could be reffered to:

1. Mucoadhesivity of alginate could increase the contact time between the microspheres and absorptive epithelium and mucosal lymphoid tissue M cells, and result in more uptake of encapsulated antigen (12,16,20).

2. Low toxicity, irritability and immunogenicity as well as biodegradability (12,16).

3. Alginate microspheres may serve not only as delivery system but also as adjuvants. Alginate can induce production of cytokines and enhance antibody responses similar to other adjuvants (20). Alginate microspheres have been used in several oral and nasal immunization studies (2,3,6,12,20,23,25).

Alginate as a vaccine delivery system could produce strong antibody responses when soluble antigens were encapsulated in and administered by nasal route (12). One of the most important characteristics of the alginate microspheres is their particle size.

Microsphere size influences the uptake by M cells (11,18) as well as the character of the immune response. Particles that are smaller than 1 μm in diameter appear to intercalate within the glycocalyx of M cells more easily than larger particles and are therefore more likely to be taken up (2). Particles smaller than 5 μm may be transferred to the draining lymph nodes and spleen and stimulate both mucosal and systemic immune responses. Particles in the range of 5-10 μm tend to remain in Peyer's patches to stimulate primarily a mucosal immune response (2). Particles larger than 10 μm are not likely to be taken up at all (9).

Among different procedures used for preparation of alginate microspheres including spraying, coacervation and emulsification, the last one is the most frequently used procedure especially for preparation of microspheres with diameter less than 10 microns (6,16,20).

Despite extensive use of alginate microspheres as a drug delivery system, formulation parameters which can affect the microspheres features, have not extensively studied.

Lemoine *et al.* (16) used an emulsification procedure (alginate in iso-octane) for preparation of alginate microspheres having a mean diameter of 10 μm or less. They studied the effects of alginate concentration and molecular weight and different kinds of surfactants on size range and aggregation of microspheres.w/o emulsification method was also used by Cho *et al.* (6) for preparation of microspheres having a diameter less than 5 μm. They evaluated the impacts of different alginate and CaCl$_2$ concentration and also different surfactants and stirring rates on microspheres size.

In the present study alginate microspheres were prepared by an emulsification method in order to deliver the tetanus toxoid (as a model antigen) to the nasal mucosa.

Different parameters influencing the size as well as morphological and release characteristics of alginate microspheres were investigated in order to prepare microspheres with mean diameters of 10 μm or less, which could be taken up by the local lymphoid tissue M cells. The effect of preparation procedure on the stability and antigenicity of encapsulated antigen (tetanus toxoid) was also investigated.

## Materials and Methods

*Materials:* Sodium alginate, bicinchoninic acid (BCA), bovine serum albumin (BSA) and Span-80 were purchased from Fluka (Buchs, Switzerland). The intrinsic viscosity of a 2% w/v solution of sodium alginate was 1050 CP as measured by a Fungilab viscometer (Fungilab, Barcelona, Spain). The ratio of mannuronic acid to gluronic acid residues (M/G) was 1.614. Calcium chloride, n-octanol, sodium citrate and isopropyl alcohol were from Merck (Darmschtadt, Germany). Tetanus toxoid solution (2500 Lf/ml) and alum-adsorbed tetanus toxoid (50 Lf/ml) were from Razi Inc. (Karaj, Iran). All chemicals were of analytical grade and were used as received.

*Preparation of alginate microspheres containing TT:* The preparation method was a modification of emulsification technique described by Cho et al (6). Briefly, 0.5-1.5 ml of aqueous solution of sodium alginate (2-4% w/v) having 5% v/v tetanus toxoid was dispersed in an n-octanol solution containing 1-3% w/v of a lipophilic surfactant (Span-80). For the primary dispersion, a mechanical homogenizer (Ultra-turrax, Ika werke, Staufen, Germany) at 8000 rpm was used. Emulsion was prepared by probe sonication (Soniprep-150, MSE, Sussex, UK) in an amplitude of 18 for 30-90 seconds.

The prepared W/O emulsion was rapidly added to a solution (30-90 ml) of calcium chloride in octanol (0.25-0.5% w/v) while stirring the whole medium slowly with a

magnetic stirrer. After 10 min for further hardening of microspheres, 2 ml of isopropyl alcohol was added dropwise. The microspheres were collected by filtration, washed with 15 ml of isopropyl alcohol and dried in a vacuum desicator for overnight. In order to prepare alginate microspheres with a diameter of 10 μm less, the effects of various operational and formulation factors on the size and morphological characteristics of microspheres were investigated. 11 batches of microspheres were prepared and marked as B-1 to B-11 (table 1). For each studied variable, batches of microspheres were prepared in triplicate.

***Determination of the encapsulation rate of tetanus toxoid (TT) in alginate microspheres :*** To determine the TT encapsulation efficiency in alginate microspheres, 5 mg of TT containing microspheres were dissolved in 750 μl sodium citrate (0.1 M pH 7.4) by shaking at room temperature for 3 hours. Bicinchoninic acid (BCA) and Bradford protein assays were used to determine the TT concentration in the microsphere solutions. Bovine serum albumin (BSA) was used as standard protein. Since sodium alginate solution per se showed some absorbance in these methods, in all assays, absorbance of a solution of empty microspheres was also read and subtracted from the absorbance of TT containing microspheres.

From these results, the amount of TT entrapped per dry weight of microspheres (TT loading) and the percentage ratio of actual TT content (w/w) to theoretical TT content (w/w) (encapsulation rate) were determined. For each batch of microspheres the encapsulation efficiency was determined in triplicate.

***Morphology and size analysis of alginate microspheres:*** Optical microscope (Carl Zeiss, Oberkochen, FRG) was used for both studying the morphological aspects of microspheres and analysing the size distribution. For the latter purpose, the diameter of 300 microspheres was determined under the optical microscope equipped with an eyepiece reticule.

Surface roughness, surface porosity, clumping and sphericity of microspheres were regarded as criteria for comparison between different batches. Three hundred microspheres of each batch were studied under optical microscope and the above mentioned characteristics were quantitated using a numbering sterategy (table 2).

The volume mean diameter of microspheres was determined by a particle size analyzer (Zetasizer 2000, Malvern, UK).

**Structural stability and antigenicity of encapsulated TT**

***Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE):*** The molecular intacticity of encapsulated TT was determined by SDS-PAGE method. Solutions of TT containing microspheres in sodium-citrate, original TT and a molecular weight reference marker were loaded onto 10% acrylamide gel and run using electrophoresis system (paya-pajoohesh, Iran). Protein bands were visualised by commassie blue and silver nitrate staining.

***Enzyme linked immunosorbant assay (ELISA):*** The TT-containing alginate microspheres were dissolved in 0.1 M citrate solution (pH 7.4). The amount of TT in this solution was determined by micro-BCA protein assay and immunoreactivity of TT was determined by an ELISA method (8). Briefly, a 96-well ELISA plate (Nunc-immunosorb, Maxisorb, Denmark) was coated with 50-1000 ng/well (100 μl of each concentration in quadruplicate) of the above mentioned TT solution and standard TT solution in phosphate buffer (0.05 M, pH 7.4) and incubated for 60 min at 37°C. After blocking the unreacted sites with 1% BSA (300 μl/well) and washing with PBS (0.05 M, pH 7.4)- Tween 20 (0.05%), 100 μl of working dilution of mice hyperimmune sera was added to each well.

The hyperimmune sera (as a source of anti-TT IgG) was from mice immunized three times by S.C. injection of 2 Lf alum-adsorbed TT. The proper working dilution of hyperimmune sera was determined by a proprietary ELISA assay. After 1 h of incubation at 37°C followed by washing, 100 µl of working dilution of goat anti-mouse IgG conjugated to horseradish peroxidase was added to each well and the plate was again incubated for 60 min at 37°C followed by four times washing. To develop the colour, 100 µl of 3,3′,5,5′-tetramethylbenzidine (TMB, KPL): peroxidase (1:1) solution was added to each well. After stopping the reaction by addition of 50 µl/well of 1 M phosphoric acid, the absorbance was measured at 450 nm.

**In vitro release study of TT from alginate microspheres:** For in vitro release study of TT from TT loaded microspheres, 30 mg of each batch of alginate microsphere was placed into 1.5 ml tubes containing 600 µl release media (PBS containing 0.01% sodium azide). The tubes were then incubated at 37°C under continuous shaking. At selected time intervals, tubes were centrifuged at 15000 rpm for 5 min. The TT concentration in the supernatants was determined by BCA method. Each assay was performed in triplicate.

*Statistical analysis:* Statistical analysis of the results was carried out using unpaired t-test.

## Results and discussion

### Preparation of Ca-alginate microspheres and evaluation of some parameters affecting the morphological and size characteristics of microspheres

The effect of some variables including surfactant concentration, sodium alginate concentration, sonication time, $CaCl_2$ concentration and volume of $CaCl_2$ solution (Table 1) on the morphological and size characteristics of prepared microspheres were evaluated. The influence of each variable on size features (mean diameter, size range and percent of microspheres with diameter greater than 10 µm) was compared using optical microscope and lazer diffraction size analyzer. Some scanning electron microscope pictures from alginate microspheres have been presented in figure 1.

In this study all the volume mean diameter determined by size analyzer was smaller than mean diameter calculated from microscopic observations. In the optical microscopic studies since the minimum determined size on eyepiece reticule is 1 µm, microspheres smaller than 1 µm could not be measured, so in calculations all of these microspheres were regarded as 1 µm. However as the size analyzer can measure the particle sizes as small as 0.02 µm and enter them in averaging, the mean size was smaller.

Table 1. Various parameters investigated for the preparation of alginate microspheres

| Batch | Surfactant conc. (w/w%) | Alginate conc. (w/v%) | Sonication time (sec.) | $CaCl_2$ conc. (w/v%) | $CaCl_2$ solution volume (ml) |
|---|---|---|---|---|---|
| B-1 | 2 | 3 | 3×30 | 0.33 | 60 |
| B-2 | 1 | 3 | 3×30 | 0.33 | 60 |
| B-3 | 3 | 3 | 3×30 | 0.33 | 60 |
| B-4 | 2 | 2 | 3×30 | 0.33 | 60 |
| B-5 | 2 | 4 | 3×30 | 0.33 | 60 |
| B-6 | 2 | 3 | 2×30 | 0.33 | 60 |
| B-7 | 2 | 3 | 1×30 | 0.33 | 60 |
| B-8 | 2 | 3 | 3×30 | 0.25 | 60 |
| B-9 | 2 | 3 | 3×30 | 0.50 | 60 |
| B-10 | 2 | 3 | 3×30 | 0.33 | 30 |
| B-11 | 2 | 3 | 3×30 | 0.33 | 90 |

**alginate microspheres encapsulated with tetanus toxoid**

Table 2. Criteria used for the numbering of qualititative parameters

| Qualititative feature | Definition | Percent of Microspheres | Quantitative Number |
|---|---|---|---|
| Roughness | Presence of very small pores on the surface of microspheres | 0<br>1-25<br>25-50<br>50-75<br>75-100 | 0<br>1<br>2<br>3<br>4 |
| Surface porosity | Presence of large pores and cavities on the surface of microspheres | 0<br>1-3<br>3-5<br>5-8<br>8-10 | 0<br>1<br>2<br>3<br>4 |
| Clumping | Presence of aggregated microspheres | 0<br>1-10<br>10-20<br>20-50<br>50-100 | 0<br>1<br>2<br>3<br>4 |
| Sphericity | Complete sphere<br>Complete ellipsoid<br>Other intermediate numbers were chosen qualititatively | | 4<br>0<br>1-3 |

Table 3. Characteristics of TT loaded alginate microspheres prepared by different formulation parameters

| Batch | Mean diameter (μm) (microscope) | Volume mean diameter (μm) (size analyzer) | Size range (μm) | Percent over 10 μm (microscope) | Microsphere yield | Surface roughness* | Surface porosity* | Clumping* | Sphericity* |
|---|---|---|---|---|---|---|---|---|---|
| B-1 | 2.2 ± 0.1 | 1.3 ± 0.4 | 0.3 – 2.1 | 1.5 ± 0.7 | 90 ± 33 | 0 | 0 | 1 ± 0 | 3.5 ± 0.7 |
| B-2 | 1.9 ± 0.1 | 1.1 ± 0.5 | 0.3 - 2.1 | 1.1 ± 0.3 | 117 ± 16 | 1.2 ± 0.8 | 0.7 ± 0.3 | 2.3 ± 0.6 | 3.3 ± 0.6 |
| B-3 | 1.7 ± 0.3 | 1.4 ± 0.6 | 1.0 - 1.65 | 0.3 ± 03 | 140 ± 9 | 1.3 ± 0.6 | 0.7 ± 0.6 | 4 ± 0 | 3.3 ± 0.6 |
| B-4 | 1.4 ± 0.2 | 1.1 ± 0.4 | 0.3 – 2.0 | 0 | 152 ± 12 | 0.2 ± 0.3 | 0 | 0 | 2.3 ± 1.5 |
| B-5 | 3.4 ± 0.6 | 4.3 ± 2.9 | 0.5 – 7.3 | 6.5 ± 0.4 | 75 ± 25 | 1.5 ± 0.7 | 0 | 0.2 ± 0.3 | 3.5 ± 0.7 |
| B-6 | 1.9 ± 0.4 | 2.7 ± 0 | 1.6 – 3.5 | 1 ± 0.3 | 104 ± 33 | 1.3 ± 1.5 | 0.7 ± 0.6 | 2 ± 1 | 3 ± 1 |
| B-7 | 2.0 ± 0.1 | 1.6 ± 0.5 | 0.9 - 2.1 | 0.7 ± 0.5 | 141 ± 15 | 1.8 ± 1 | 1.7 ± 1.6 | 2 ± 1.7 | 2.7 ± 1.5 |
| B-8 | 1.9 ± 0.4 | 1.6 ± 0.1 | 0.8 – 2.3 | 1.8 ± 1.9 | 94 ± 28 | 2 ± 1 | 0.7 ± 0.6 | 1.8 ± 1.2 | 3 ± 0 |
| B-9 | 2.3 ± 0.6 | 2.2 ± 0.8 | 1.1 – 3.4 | 2 ± 0.1 | 145 ± 5 | 2 ± 1.7 | 0.7 ± 0.1 | 3 ± 1 | 3.3 ± 0.6 |
| B-10 | 2.2 ± 0.1 | 1.9 ± 0.2 | 1.1 – 2.7 | 1.5 ± 1.0 | 144 ± 13 | 2.7 ± 2.3 | 0 | 2.7 ± 2.3 | 3 ± 0 |
| B-11 | 2.4 ± 0.4 | 2.0 ± 0.5 | 0.7 – 4.4 | 2.1 ± 1.9 | 109 ± 33 | 1.3 ± 1.5 | 0 | 1.3 ± 1.1 | 3.7 ± 0.6 |

* 0 = no, 1= low, 2 = medium, 3= high, 4= very high

Table 4. Relation between clumping and microsphere yield among microspheres made with 3% w/v alginate solution

| Batch | B-1 | B-11 | B-8 | B-6,7** | B-2 | B-10 | B-9 | B-3 |
|---|---|---|---|---|---|---|---|---|
| Clumping* | 1 | 1.3 | 1.8 | 2 | 2.3 | 2.7 | 3 | 4 |
| Microsphere yield (%) | 90 | 109 | 94 | 123 | 117 | 144 | 145 | 140 |

* 0 = no, 1= low, 2 = medium, 3= high, 4= very high

** B-6+B-7/2

Table 5. Encapsulation rate of TT in different microsphere batches and amount of TT released (µg) in the first hour and in one week

| Batch | B-1 | B-2 | B-3 | B-4 | B-5 | B-6 | B-7 | B-8 | B-9 | B-10 | B-11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| TT Encapsulation rate (%) | 47.7 | 41.6 | 29.2 | 26.2 | 25.6 | 26.2 | 28.8 | 34.3 | 34.6 | 29.6 | 32.4 |
| SD (n=3) | 6.6 | 11.7 | 2.7 | 5.0 | 6.9 | 3.7 | 9.2 | 3.2 | 6.8 | 9.0 | 7.0 |
| Release in first hour | - | 6.2 | 3.4 | 3.2 | 2.6 | 3 | 3.2 | 3.2 | 1.2 | 0 | 4.8 |
| Release after one week | - | 29.8 | 27.1 | 24.4 | 25.2 | 25.6 | 25 | 31.3 | 21.4 | 17.6 | 27.8 |
| 1h / 1w ratio (%) | - | 21 | 12.5 | 13.2 | 10.3 | 11.7 | 12.8 | 10.2 | 5.6 | 0 | 17.3 |
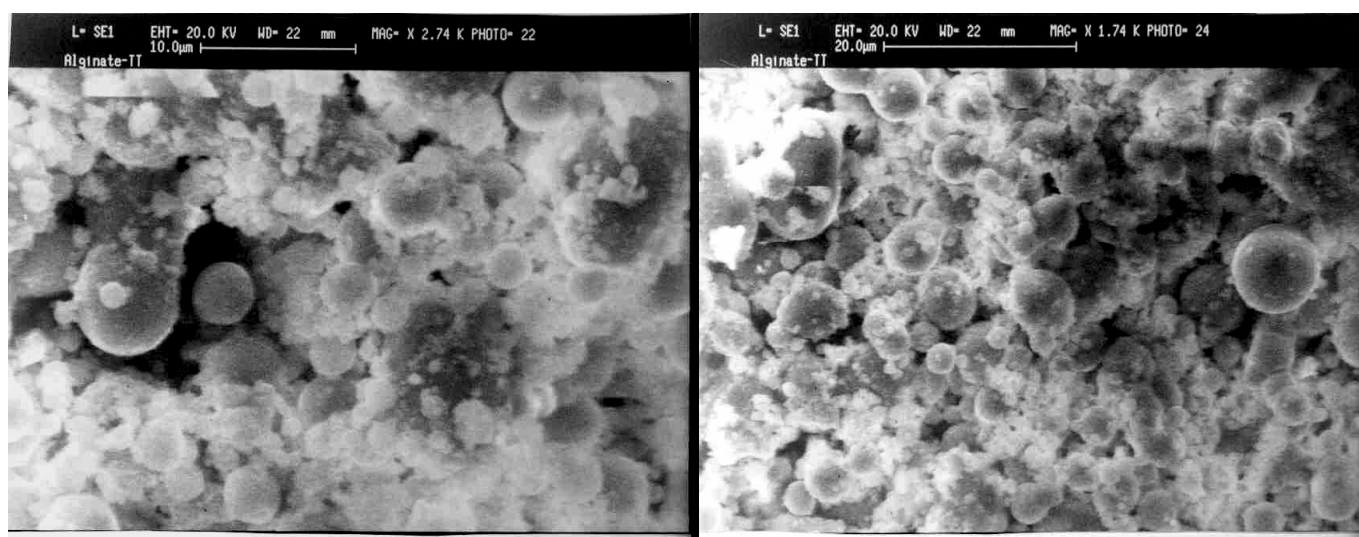


Figure 1: Scanning electron microscope pictures from alginate microspheres (B-1 batch)
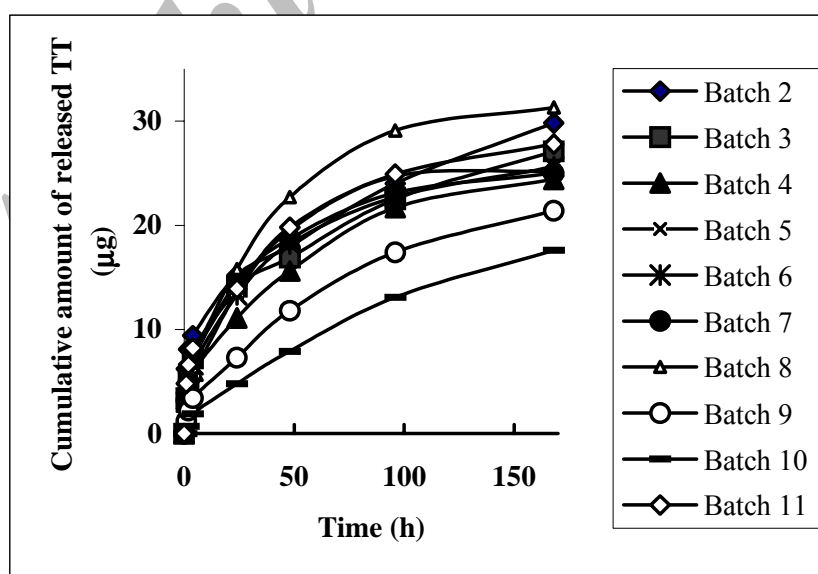


Figure 2. Cumulative amount (µg) of tetanus toxoid released from different batches of alginate microspheres in one week

**Effect of surfactant concentration**

Sorbitan monooleate (Span-80) was used as emulsifier for the emulsification of sodium alginate solution in octanol. Span-80 was dissolved in octanol at concentrations of 1, 2 and 3% w/v (Batches B-2, B-1 and B-3, table 1). Each batch was made in triplicate and the influence of each concentration on the characteristics of microspheres was studied (Table 3).

The mean diameter of microspheres made with 1-3% of surfactant were the same (P>0.05). Percent of microspheres over 10 μm in B-3 batch is significantly lower than the other two batches (P<0.05). Regards to the size range and percent of microspheres over 10 μm, B-3 showed a considerably more uniform distribution than the B-1 and B-2.

Microspheres made with 2% surfactant showed the best morphological characteristics that is; no roughness, no porosity, low clumping and high sphericity. Regards to the microspheres made with 3% surfactant, clumping was very high. It seems that by increasing the surfactant concentration from 2% to 3%, some very small microspheres are clumping together and this could be the reason why microspheres under 1 μm were not obtained in B-3.

**Effect of sodium alginate concentration**

There are different reports on the optimum concentration of sodium alginate for preparation of smooth and discrete microspheres. Cho et. al. (6) by introducing the 5% w/v as the optimum concentration, reported that at higher concentrations it was difficult to produce small, homogeneous microspheres, while at lower concentrations agglomerated microspheres were obtained. Lemoine et. al. (16) also proposed the 5% w/v as the optimum, while Wan et. al. (24) prepared the best microspheres by 2-3% w/v alginate concentrations.

In this study, after a preliminary test (data not presented), it appeared that due to high viscosity of the 5% and higher concentrations

of alginate solutions, these high concentrations can not give the proper microspheres. Three concentrations of 2, 3 and 4% w/v (Batches B-4, B-1 and B-5, table 1) were used and their impacts on size and morphological characteristics of microspheres were studied (Table 3).

It has been shown that by increasing both the alginate concentration and molecular weight which would result in higher viscosity of solutions, the microsphere size would also increase (14,16).

In the present study while mean diameters obtained with optical microscope are in accordance with the above reports (B-5>B-1>B-4, P<0.05), mean diameters obtained from size analyzers are not significantly different (P>0.05). Alginate concentration had an obvious effect on the size range such that by increasing the concentration from 2% to 4%, a higher percentage of over 10 μm microspheres (B-5>B-1>B-4, P<<0.05) and wider size range (B-5>B-1=B-4) were resulted. Surface roughness for 2 and 3% batches was very low but for 4% alginate solution more roughness was observed which could be resulted from higher viscosity of 4% alginate solution. Microspheres made with these three concentrations of sodium alginate showed little or no aggregation. It seems that this parameter is not affected by alginate concentration.

In 3% and 4% batches, sphericity was high, but in 2% batch sphericity was low. It could be assumed that the low viscosity of 2% solution of alginate is the reason. When emulsified alginate is added to rotating solution of $CaCl_2$ in octanol, because of low viscosity of alginate droplets, shear stress resulting from the rotating solution will elongate the droplets before solidification of alginate droplets to form microspheres.

**Effect of sonication time**

Since the microemulsified alginate droplets form microspheres, the emusification procedure and resulting

emulsion have important effects on morphological features of final microspheres. Cho et. al. (6) have reported that stirring rate of homogenizer has an important role in forming the microemulsion droplets with the desired diameter, prior to gelation by $CaCl_2$. These authors have shown that in a constant time, in stirring speeds less than 5000 rpm, heterogeneous microspheres larger than 10 µm were obtained, while at speeds higher than 8000 rpm, agglomerated particles with a low yield were formed. They suggested the optimum speed to be 8000 rpm for 1 h.

In the present study by replacing homogenizer with probe sonicator for emulsification, the effect of different sonication times (Batches B-7, B-1 and B-6, table 1) on prepared microspheres was evaluated (Table 3).

It seems that in this time range, sonication time doesn't have any significant effect on the mean diameter (microscope), percent over 10 µm, microsphere yield (P>0.05) and size range.

However sonication time had an obvious influence on morphological characteristics of microspheres. It seems that in emulsification of alginate by means of sonication, stronger ultrasound waves will treat the surface of alginate droplets and will result in less roughness and porosity as well as more sphericity .

### Effect of CaCl₂ concentration

When emulsified sodium alginate is added to a solution of $CaCl_2$ in octanol, the carboxylate groups of the gluronate monomers of alginate are complexed with calcium cations. This networking shrinks the microspheres and reduces the space occupied by alginate droplets, and therefore decreases the volume and diameter of the microspheres (7).

In this study, effect of three concentrations (0.25, 0.33 and 0.5% w/v) of $CaCl_2$ in octanol (Batches B-8, B-1 and B-9, table 1) on microsphere size and morphological characteristics were evaluated (Table 3). It was found that in these concentrations of calcium, mean diameters (microscope and size analyzer), percent over 10 µm (P>0.05) and size range of different batches were not significantly different. However regarding the morphological characteristics, the most optimum calcium concentration was found to be 0.33% w/v.

Microspheres made with this concentration had no roughness, no porosity, low clumping and high sphericity. An interesting finding was the high clumping of microspheres made with 0.5% $CaCl_2$. In preliminary studies (data not shown), higher concentrations of $CaCl_2$ (up to 3% w/v) were also examined, but in all of those batches, microspheres were highly aggregated.

It seems that at higher concentration of calcium, outer gel layer will form rapidly resulting in higher stickness and thus more aggregation of microspheres.

### Effect of volume of CaCl₂–in-octanol solution

Three different volume of $CaCl_2$–in-octanol solution (Batches B-10, B-1 and B-11, table 1) were evaluated. While this parameter didn't show a significant effect on mean diameters (microscope and size analyzer), percent over 10 µm, microsphere yield (P>0.05) and size range of microspheres, it affected the morphological characteristics of microspheres (Table 3).

Regarding all the examined parameters, the B-1 batch was the most optimum one. Microspheres made with 30 ml $CaCl_2$ solution showed the highest roughness and clumping and lowest sphericity. It seems that in a constant volume of alginate in octanol emulsion added to gelation media ($CaCl_2$ in octanol solution), decreasing the volume of gelation medium will result in more collision between microemusified alginate droplets and resulting microspheres. This higher collision could be the reason for higher roughness of microspheres surface and less sphericity as well as more clumping.

Another parameter which can affect the microspheres by increasing the collision, is the stirring rate of the gelation medium. In our preliminary studies (data not shown), we found that stirring rate of gelation medium had an important role in morphological features and specially on clumping of microspheres. It seems that by increasing the stirring rate, higher collision between microemulsified alginate and formed microspheres will result and more clumping will occur. The best condition for getting discrete and smooth microspheres is to keep the stirring at the lowest rate needed for a moderate circulation of media. This has also been advised by some other reseachers (6).

**Microsphere yield**

The ratio of the weight of desicated alginate microspheres to dry weight of sodium alginate powder used for preparation of microspheres was taken as microsphere yield.

Considering the microsphere yield of different batches (Table 3), two questions will arise.

First, what's the reason for low microsphere yield of the batch B-5?

It was found that in emusification of 4% alginate solution (batch B-5), possibly due to the high viscosity of alginate solution, some amount of alginate remained unemulsified. This was also the case with higher concentrations of alginate solution used in other preliminary studies (data not presented). In batches made with 5% alginate solution, a microsphere yield of 30% was achieved and a high amount of alginate solution was remained unemusified.

Second what's the reason for microsphere yield of over 100%? Considering the different microspheres made at various conditions (Table 3), it seems that the over 100% microsphere yield is only proportional to the degree of clumping of microspheres (Table 4). This can be explained as follows:

In phenomenon of synersis which occure in the crosslinking of alginate with $Ca^{++}$, water is diffusing out and microsphere weight and volume decreases (7).

When microspheres are aggregated, the diffuse out of water from internal microspheres will be decreased and some water will remain inside the microsphere aggregate, even after desication. The remaining water could be the reason for microsphere yield of over 100%. It has been shown in Table 4 that by increasing of microspheres clumping from medium to very high, the microsphere yield increased from nearly 100% to 140%.

**Encapsulation efficiency of tetanus toxoid (TT) in alginate microspheres**

Encapsulation rate of TT in different batches of microspheres was determined (Table 5) and the results were compared using one way ANOVA. While encapsulation rates in B-2 to B-11 are not significantly different (P>0.05), encapsulation rate of B-1 was significantly greater than that of B-3 to B-7 and B-10 (P<0.05).

**Effect of preparation variables on in vitro antigen release**

In vitro release of encapsulates from alginate microspheres can be affected by several factors. It has been shown that release rate of low molecular weight drugs increases by increasing the manurunic/glurunic acid ratios (17,21,22). Kikuchi et. al. (15) have also shown that the release of dextran from alginate beads is a molecular weight-dependent process; release for low molecular weight dextrans is diffusion controlled but for higher molecular weight dextrans, it is controlled by alginate gel disintegration process.

Dextran release is also influenced by the exchange of $Na^+$ ions in PBS with $Ca^{++}$ ions bound to carboxylates in the alginate molecules; decreasing the NaCl concentration, reduces the exchange rate of

$Na^+$ ions with $Ca^{++}$ ions. This should affect the dissolution of alginate, and thus, the release of high molecular weight dextran (15).

Another parameter which affect the release from alginate gels is cation valency and radius. Al-Musa et al. (1) have shown that in cross-linking of sodium alginate gel beads, the trivalent cations like $Al^{3+}$ are expected to form a three dimensional valent bonding structure with the alginate. In this structure, all the aluminium ions are able to diffuse into the surface gel layer of alginate beads before complete cross-linking of the surface occurred. In the case of divalent cations like $Ca^{2+}$, their plannar two dimensional bonding to alginate can decrease the penetration of Ca cations and will lead to a lower cross-linking. It has also been shown that the release from Al-alginate is slower than that of Ca-alginate gel beads (1).

As it is demonstrated in figure 2, all batches of microspheres (B2-B11) made at various conditions (Table 1) show simillar pattern of release. As a criterion for expressing the initial release of TT from alginate microspheres, the ratio of TT released in the first hour to that released in one week was compared (Table 5). With regard to the high internal porosity of alginate microspheres and beads, the initial release have been normally very high; in a manner that in some expriments 90% of encapsulate released in 15 min (4).

In this study the amount of encapsulate released in the first hour ranges from 5 to 21% of the total release in one week. This can be regarded as a criterion for lower porosity of microspheres prepared by this procedure.

Comparing the two batches of B-2 and B-3 which were made with different percentages of surfactants (Table 2), B-3 showed a lower initial release than that of B-2. Referring to Table 2, the main difference between these two batches is the higher degree of clumping in microspheres of B-3. It seems that higher clumping decreases the contact area of microspheres with release media resulting in lower initial release.The same explanation is applicable for comparison between batches B-4 and B-5, B-8 and B-9 and also B-10 and B-11. The total amount of TT released from microspheres in one week ranges between 10-30 μg.

**Structural intacticity and antigenicity of tetanus toxoid after encapsulation process**

An important prerequisite for a delivery system of antigens is the preservation of their intact structure and specially their antigenicity. In the process of encapsulation of TT in microspheres, the antigen is exposed to several potentially harsh conditions such as contacting with organic solvents, surfactants and sonication.Such conditions would potentially affect the intacticity or antigenicity of antigens. Therefore the molecular stability of tetanus toxoid encapsulated in the most optimum batch of microspheres (B-1) was determined using SDS-PAGE. In SDS-PAGE gels, identical bands were observed for the original TT and TT encapsulated in microspheres, showing an intact TT in the microspheres.

An ELISA method was used to determine the antigenicity of TT encapsulated in alginate microspheres. The antigenicity of encapsulated TT was checked by coating the ELISA plate with 50,100,250,500 and 1000 ng of encapsulated TT (in triplicate) in the presence of the same amounts of native TT (in quadruplicate).In this assay the antigenicity was calculated as 91±5.2%.

Therefore it was concluded that in the preparation method used in this study, the harsh conditions can affect the antigen and decrease its immunoreactivity to about 9%, even though these conditions had no effect on the structure of tetanus toxoid.

**Conclusion**

Alginate microspheres with optimum size and morphological characteristics were prepared.

The most optimum microspheres (B-1) had a volume mean diameter of 1.34 μm; without any surface roughness and porosity; low clumping, very high sphericity and a high encapsulation rate.

Microspheres made with 2% w/v surfactant (Span-80), 3% w/v sodium alginate solution, 90 seconds sonication and gelled in 60 ml of 0.33% w/v $CaCl_2$ in octanol (B-1 batch, Table 1) showed the most desirable characteristics and highest encapsulation rate (47.7%) among different batches.

The release profile of tetanus toxoid as a model antigen from different batches was studied. 5-21% of the total release in one week, was released in the first hour which can be regarded as the burst effect.

The preparation procedure didn't affect the structure of tetanus toxoid which used as model antigen but reduced its antigenicity by about 9% of the original TT.

It is therefore concluded that alginate microspheres made by emulsification method could be regarded as appropriate vhicle for the delivery of TT via nasal route.

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